

# *Cannabidiol (CBD) improves survival and behavioural comorbidities of Dravet syndrome in mice*

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## RESEARCH PAPER

# Cannabidiol improves survival and behavioural co-morbidities of Dravet syndrome in mice

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**Background and Purpose:** Dravet syndrome is a severe, genetic form of paediatric epilepsy associated with premature mortality and co-morbidities such as anxiety, depression, autism, motor dysfunction and memory deficits. Cannabidiol is an approved anticonvulsive drug in the United States and Europe for seizures associated with Dravet syndrome in patients 2 years of age and older. We investigated its potential to prevent premature mortality and improve associated co-morbidities.

**Experimental Approach:** The efficacy of sub-chronic cannabidiol administration in two mouse models of Dravet syndrome was investigated. The effect of cannabidiol on neonatal welfare and survival was studied using *Scn1a*<sup>-/-</sup> mice. We then used a hybrid, heterozygote *Scn1a*<sup>+/-</sup> mouse model to study the effect of cannabidiol on survival and behavioural co-morbidities: motor deficits (rotarod and static-beam test), gait abnormality (gait test), social anxiety (social interaction test), anxiety-like (elevated plus maze) and depressive-like behaviours (sucrose preference test) and cognitive impairment (radial arm maze test).

**Key Results:** In *Scn1a*<sup>-/-</sup> mice, cannabidiol increased survival and delayed worsening of neonatal welfare. In *Scn1a*<sup>+/-</sup> mice, chronic cannabidiol administration did not show any adverse effect on motor function and gait, reduced premature mortality, improved social behaviour and memory function, and reduced anxiety-like and depressive-like behaviours.

**Conclusion and Implications:** We are the first to demonstrate a potential disease-modifying effect of cannabidiol in animal models of Dravet syndrome. Cannabidiol treatment reduced premature mortality and improved several behavioural co-morbidities in Dravet syndrome mice. These crucial findings may be translated into human therapy to address behavioural co-morbidities associated with Dravet syndrome.

**Abbreviations:** AED, Anti-epileptic Drug; ASPA, Animals (Scientific Procedures) Act; CBD, cannabidiol; TNW, Total Neonatal Welfare.

Claire Michelle Williams and Alister James McNeish contributed equally.

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## 1 | INTRODUCTION

Dravet syndrome is a severe form of myoclonic epilepsy in children (Dravet, Bureau, Oguni, Fukuyama, & Cokar, 2005). It is typically triggered by a fever which initiates the first generalised or partial seizure at usually 6 to 9 months of age (Dravet et al., 2005). During the first year of life, seizures are relatively infrequent but over time, can lead to *status epilepticus*. In subsequent years, the symptoms become more severe with generalised tonic-clonic, myoclonic, absence and focal seizures being common, status epilepticus rarely occurs after 10 years of age (Akiyama, Kobayashi, Yoshinaga, & Ohtsuka, 2010). The *SCN1A* gene encodes *Na<sub>v</sub>1.1*, a voltage-gated sodium channel, which plays a pivotal role in Dravet syndrome where 70–80% of patients exhibit a deletion or mutation (truncating, missense, or splice site mutations leading to loss of function) of this gene on chromosome 2q (Harkin et al., 2007; Marini et al., 2011).

In addition to seizures, premature mortality occurs in approximately 21% Dravet syndrome patients (Dravet et al., 2005; Genton, Velizarova, & Dravet, 2011). Patients also exhibit multiple co-morbidities including psychomotor delay, gait abnormality, hyperactivity, attention deficits, autism, sleep disorders, anxiety, depression, language impairment and severe cognitive deficits, profoundly affecting quality of life (Genton et al., 2011). The current standard treatment includes the combination of two or more antiepileptic drugs, such as benzodiazepines, *valproic acid*, *topiramate*, *levetiracetam* and *stiripentol* (Devinsky et al., 2017). However, these drugs often fail to adequately control seizures and are associated with severe drug-induced motor and psychiatric adverse effects including anxiety, depression and memory impairments (Chen et al., 2017; Ristić, Vojvodić, Janković, Sindelić, & Sokić, 2006).

**Cannabidiol** (CBD) is one of the most abundant plant-derived cannabinoids. It is non-euphoric and has shown potential for use in treating seizures, epilepsy, epileptogenesis and related neuroprotection in a number of animal models including Dravet syndrome (Kaplan, Stella, Catterall, & Westenbroek, 2017; Patra et al., 2019; Rosenberg, Patra, & Whalley, 2017). CBD was demonstrated to be an effective anticonvulsant in Phase 3 clinical trials for the treatment of Dravet syndrome and Lennox Gastaut Syndrome and in 2018 received FDA approval in the United States for seizures associated with these disorders (Devinsky et al., 2017; FDA, 2018; Thiele et al., 2018). However, little is known about its potential effect on the premature mortality and co-morbidities associated with Dravet syndrome.

Here, we first evaluated the effect of CBD on survival and neonatal welfare in *Scn1a*<sup>-/-</sup> knockout mice. *Scn1a*<sup>-/-</sup> mice are obtained from mice with a 129S background. They differ from human Dravet syndrome by their complete loss of expression of *Na<sub>v</sub>1.1* channels, but they reproduce the ataxia, seizures and premature mortality commonly observed in Dravet syndrome patients (Kalume, Yu, Westenbroek, Scheuer, & Catterall, 2007; Yu et al., 2006). They exhibit symptoms such as seizures, ataxia and a poor righting reflex from post-natal day 9 (P9) onwards which become progressively

### What is already known about this subject

- Dravet syndrome is associated with premature mortality, seizures and associated co-morbidities.
- Cannabidiol's (CBD's) anticonvulsive action in Dravet syndrome has been demonstrated in preclinical and clinical studies.

### What this study adds

- Chronic CBD administration reduces premature mortality in two mouse models of Dravet syndrome.
- This study demonstrates that CBD improves co-morbidities in a mouse model of Dravet syndrome.

### What is the clinical significance

- CBD may improve survival in human Dravet syndrome patients.
- CBD may be a suitable treatment to reduce the possibility of behavioural co-morbidities in human Dravet syndrome patients.

worse over time, with animals typically dying before they reach P16 (Kalume et al., 2007; Miller, Hawkins, McCollom, & Kearney, 2014; Yu et al., 2006). Due to the extreme severity and early mortality, this model is not suitable for studying complex behavioural co-morbidities but does allow rapid assessment of antiepileptic drug (AED) efficacy and was used here as a proof-of-concept for CBD efficacy.

We then conducted a study in the hybrid heterozygote *Scn1a*<sup>+/-</sup> mouse Dravet model to investigate the effect of chronic CBD treatment in prevention of premature mortality and a range of complex behavioural co-morbidities such as motor impairment, anxiety, depression, social and memory deficits. This model of Dravet syndrome uses a hybrid heterozygote *Scn1a*<sup>+/-</sup> mouse model breeding male heterozygotic mice from a 129S background with female *wild-type* (WT) mice from the C57/Black six (C57/B6) background. The resultant heterozygote offspring recapitulate several features of Dravet syndrome including seizures, premature mortality and co-morbidities such as social deficit, anxiety and memory impairments (Han et al., 2012; Yu et al., 2006). They are considered a standard model to study drug effects on Dravet syndrome and complex behavioural co-morbidities (Anderson, Hawkins, Thompson, Kearney, & George, 2017; Hawkins et al., 2017; Kaplan et al., 2017).

## 2 | METHODS

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology* and UK Home Office regulations (Animals [Scientific Procedures] Act, 1986;

ASPA) under licence 70/8397 "Mouse Model of Dravet Syndrome" and were approved by the Animal Welfare and Ethics Review Board at the University of Reading (McGrath & Lilley, 2015). The study used a total of 182 mice: 95 in Study I (19 for the development of the survival algorithm, 36 for breeding/colony maintenance and 40 for the study) and 87 in Study II (15 for breeding/colony maintenance, 52 in test groups, and a further 20 WT "novel" animals for social interaction test). All mice were maintained in 12 hr:12 hr light:dark cycle, a room temperature of 21°C and humidity of 50 ± 10%, with *ad libitum* access to food and water. Both male and female mice were used in all experimental groups.

## 2.1 | Study I: Assessment of neonatal welfare and survival in *Scn1a*<sup>-/-</sup> mice

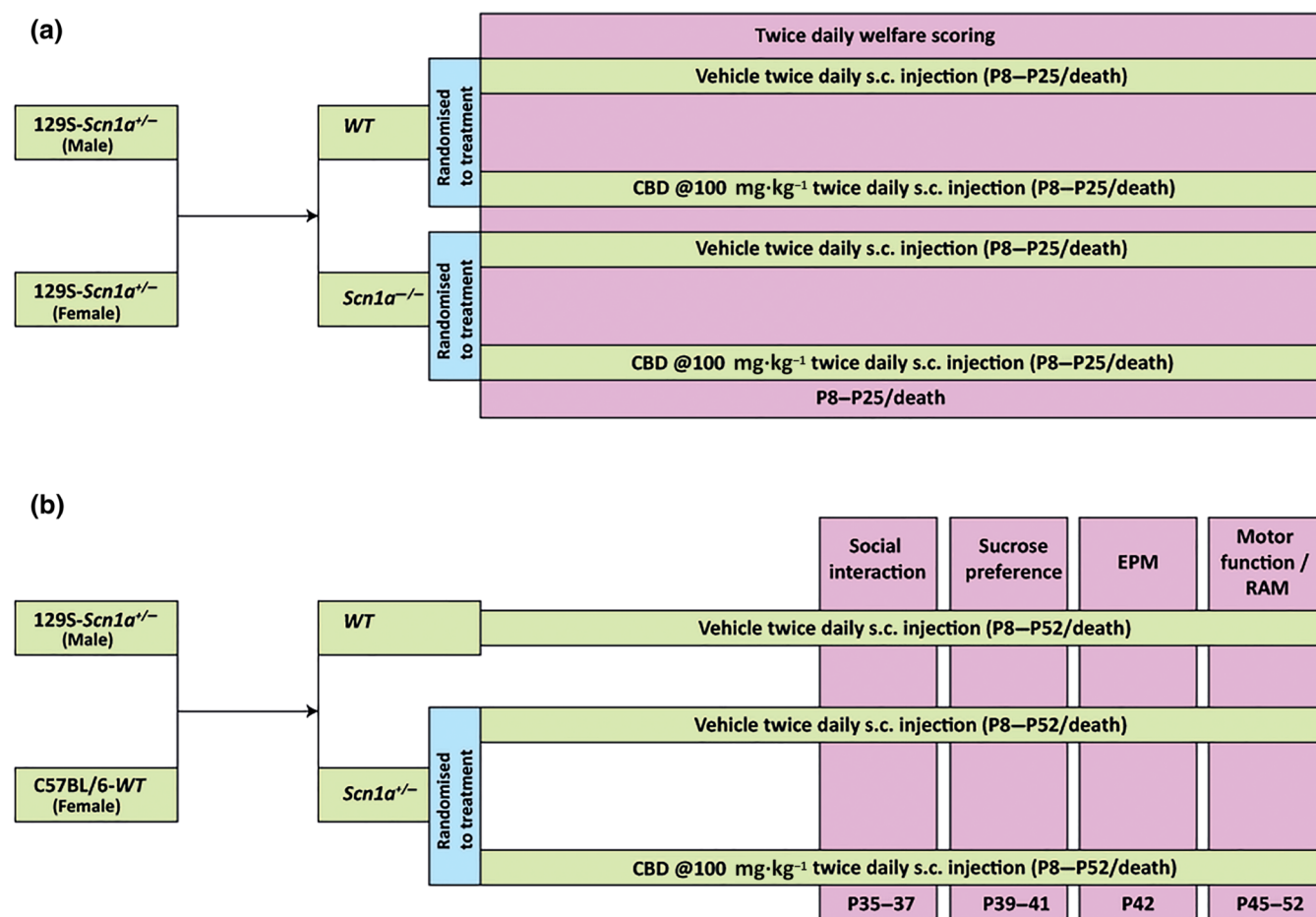
### 2.1.1 | Animals

129S-*Scn1a*<sup>tm1Koa/Mmjax</sup> (RRID:MMRRC\_037107-JAX) heterozygote mice (Jackson Laboratory, USA) were maintained in the

BioResource Unit at the University of Reading (UK) and bred together to obtain *Scn1a*<sup>-/-</sup> and WT animals used for this study (*n* = 10 per group; Figure 1a). All experiments were conducted during light phase 8:00 a.m.–8:00 p.m. The maternal behaviour (see Table S1) of the dams was also assessed simultaneously to ensure that any of the parameters observed in the study animals (*Scn1a*<sup>-/-</sup>/WT mice) were not affected by the dam's behaviour. In this study, dam scores remained 0 throughout the study and so the responses of the pups were not considered to have been affected by variations in maternal behaviours. At the end of the study, animals were humanely killed by a Schedule 1 method (cervical dislocation).

### 2.1.2 | Experimental design

Following genotyping (see supplementary material 1), animals were randomly divided into four groups: WT vehicle treated, WT CBD treated, *Scn1a*<sup>-/-</sup> vehicle treated, and *Scn1a*<sup>-/-</sup> CBD treated



**FIGURE 1** Schematic showing the timeline of the drug administration and behavioural tests. (a) Timeline of drug administration and welfare scoring in Study I. The wild-type (WT) and *Scn1a*<sup>-/-</sup> mice were injected (s.c.) twice daily with either vehicle or CBD (100 mg·kg<sup>-1</sup>) from post-natal day 8 (P8) to P25/death. Twice daily welfare scoring was conducted in all the groups throughout the experimental period. (b) Timeline of drug administration and co-morbidity tests in Study II. The *Scn1a*<sup>-/-</sup> mice were injected (s.c.) twice daily with either vehicle or CBD (100 mg·kg<sup>-1</sup>) from P8 to P52/death. A WT vehicle-treated control group was also taken. Social interaction (P35–P37), sucrose preference (P39–P41), elevated plus maze (EPM; P42) and motor function and radial arm maze (RAM; P45–P52) tests were conducted in all the groups

( $n = 10$  per group). They were injected subcutaneously twice daily with either CBD ( $100 \text{ mg}\cdot\text{kg}^{-1}$ ) or its vehicle (ethanol: Kolliphor®: 0.9% saline = 2:1:17) from P8 until P25 or death (whichever was earlier). A twice daily welfare check was conducted throughout the entire duration of the study. Drug administration was conducted at 08:00 a.m. and followed by welfare checks. Conversely, afternoon welfare checks were conducted from 04:00 p.m. and followed by drug administration in order to provide the maximum possible time between doses. The experimental timeline is depicted in Figure 1a.

### 2.1.3 | Assessment of welfare scores

Welfare scoring of neonates was conducted twice daily using a blinded spreadsheet that lacks the information on the genotype of the animals and the treatment (CBD/vehicle) given to them, to ensure the experimenter remained blind to both treatment and genotype. Neonatal welfare scoring (Table S2) was based upon a previously validated standardised approach used widely in murine models (Langford et al., 2010; Ullman-Culleré & Foltz, 1999; Wolfensohn & Lloyd, 2007). Here, the parameters used for the welfare assessment were weight, natural activity (NA; 0–3), reflex/response to touch (RT; 0–3), orbital tightening (OT; 0–2), body condition (BC) score (1–3) and surface temperature (ST; 0–2; data not shown). Finally, a total neonatal welfare (TNW) score (range 0–8) was calculated by adding together scores from NA, RT and ST. CBD treatment significantly delayed the worsening of NA, RT, OT, BC and TNW scores in the *Scn1a*<sup>−/−</sup> mice compared to their vehicle-treated counterparts. For clarity only, the TNW data have been reported in this manuscript; the rest of the welfare data have been supplied in Figure S2.

### 2.1.4 | Assessment of survival

Animal suffering was minimised by employing a validated, welfare scoring system (Table S2) alongside a mathematical model to predict death; see Data S2 and Data S3 for the original welfare data used to construct the model (Animals 1–19 only) and the welfare data for animals used in the study. In this way, any animal for which the model predicted death could be killed 0.5 day before enduring the maximal severity of the disease and enabling us to comply with welfare requirements of ASPA. The model used an algorithm to predict death based on prior data obtained from untreated *Scn1a*<sup>−/−</sup> mice ( $n = 19$ ) that exhibited the maximum severity of the disease and died a natural death (Data S3). In this algorithm, the thresholds for each parameter (TNW, NA, RT, OT and BC scores) to predict death were obtained using the following procedure: (i) Each parameter, measured every half day from birth for each animal, is averaged with a moving mean with a 1.5-day window; (ii) the least severe score for each parameter observed across the 19 animals over 0.5 day before their death was found; (iii) each of the five parameters exhibited by the animals in the

study was compared to scores obtained in (ii) twice a day; (iv) if each of the five parameters reached their respective threshold defined in (ii) at least once since P8, the animal would undergo a Schedule 1 procedure (cervical dislocation) within 0.5 day. Additionally, ST threshold was employed such that if the sum of the ST scores over the last 1.5 days was equal to or greater than 3, the animal would be killed by Schedule 1 procedure (cervical dislocation) within 0.5 day.

## 2.2 | Study II: Assessment of survival and comorbidities in *Scn1a*<sup>+/-</sup> mice

### 2.2.1 | Animals

The animals were group housed throughout the study except for 3 days during sucrose preference test when each animal was individually housed. This study was conducted in a reversed dark:light cycle with all experiments performed in the dark cycle (dim red light, 8:00 a.m.–8:00 p.m.). Male 129S-*Scn1a*<sup>tm1Koa/Mmjax</sup> heterozygote mice (Jackson Laboratory, USA) maintained in the Bio-Resource Unit, University of Reading (UK), were bred with female WT C57BL/6 mice (Charles River, UK; RRID:MGI:5656552) to obtain *Scn1a*<sup>+/-</sup> and WT littermate mice used in this study. At the end of the study, animals were humanely killed by a Schedule 1 method (cervical dislocation).

### 2.2.2 | Experimental design

Here, hybrid *Scn1a*<sup>+/-</sup> of both sexes was randomly divided into two groups and subcutaneously injected with either CBD ( $100 \text{ mg}\cdot\text{kg}^{-1}$  twice daily;  $n = 12$ ) or its vehicle (ethanol: Kolliphor®:0.9% saline = 2:1:17;  $n = 29$ ) from P8 onwards until P52 or death (whichever was earlier). Similarly, WT littermate mice ( $n = 11$ ) were injected with vehicle for the entire period of the study. Given that a significant number of deaths (~60%) were predicted to occur between P20–P27 in vehicle-treated *Scn1a*<sup>+/-</sup>, a larger initial group size was utilised to obtain a minimum  $n = 10$  animals per group for behavioural assessment from P35 onwards. Of note, we accounted the possibility that CBD-treated animals might also die; therefore, in the first run, equal group sizes were taken. However, the mortality was higher in the vehicle-treated *Scn1a*<sup>+/-</sup> group than in the CBD-treated *Scn1a*<sup>+/-</sup> group, so in subsequent runs, a greater number of animals were included in the vehicle-treated *Scn1a*<sup>+/-</sup> group. The experimental timeline has been depicted in Figure 1b.

### 2.2.3 | Assessment of survival

As seizure-related deaths in this model were unpredictable, animals were video monitored continuously (24 hr × 7 days) throughout the study, and any mortality observed was cross-checked with the

available video footage (1 hr prior to death) to confirm the reason of death.

## 2.2.4 | Assessment of motor function

Fine motor control in animals were assessed by the accelerated rotarod and static beam tests. Animals were habituated to the stationary rotarod for 2 min a day for 2 days. In the accelerated rotarod test, each mouse was placed individually on a linearly accelerating rod (4–40 rpm over 5 min; LE8500, Letica Scientific Instruments, UK), and average latency to fall from the rod (maximum 300 s) was calculated from three consecutive trials (2 min interval between trials).

The static beam task was further employed to analyse balance and coordination (Sedy, Urdzikova, Jendelova, & Sykova, 2008), where the animals were required to walk along a cylindrical elevated beam (100 cm long, 0.9 cm diameter, and 50 cm height from floor) and enter a dark enclosure at the beam end. The mice were habituated to the task for three consecutive days before the test day. Each day of the habituation period, the animals were placed 30, 60, and 100 cm away from the enclosure and allowed to traverse along the beam. On the test day, each mouse performed two consecutive trials (2-min interval between trials) with a maximum given time of 2 min to complete the task (the nose entering the box was taken as task completion). The test was video monitored (Sony DCR-SX21E), and blinded offline analysis was conducted (Observer XT 12, Noldus, the Netherlands) to evaluate the average number of foot slips made from two consecutive trials.

## 2.2.5 | Assessment of gait

Gait test was conducted to assess the cerebellar function of the animals (Patel & Hillard, 2001). In this test, the hind paws of each mouse were marked with a non-toxic ink, and the mouse was allowed to walk on a white paper (50 × 10 cm) placed on the floor of a custom-made plexiglass tunnel (50 × 10 × 10 cm). To obtain the left and right stride length, the distance between two ipsilateral paw prints was measured, whereas stride width was calculated from the distance between a foot print and its contralateral stride length at right angle (Wecker et al., 2013). The initial and last footprints were not considered in measurements. All the animals were habituated to the test procedures and the apparatus for 2 days prior to the test. On the day of test, two trials were conducted for each animal to obtain mean stride length (left or right) and width for that animal.

## 2.2.6 | Assessment of social interaction

The social interaction test was conducted in the home cage of test mouse to assess the social behaviour of the animals (Sato,

Mizuguchi, & Ikeda, 2013). On test day, cage mate(s) were removed from the home cage of the test mouse, and the mouse remained in isolation for 15 min. A novel WT mouse of same strain, same sex, and similar weight to the test mouse was then introduced to the home cage of the test mouse. Activity was video recorded (Sony DCR-SX21E) for 10 min, and the obtained video files were blinded at the end of all experiments. Time spent in active interactions (e.g. close following, sniffing, allogrooming/social grooming and mounting) and number of rearing (lifting the front paws on the air) occasions were coded offline using Observer XT 12 (Noldus, the Netherlands). Aggressive behaviours were not considered as social interactions and were not coded. In this test, a reduced social interaction is considered as autistic-like behaviour (Sato et al., 2013), while increased rearing occasions is sign of defensive escapes (Kaplan et al., 2017).

## 2.2.7 | Assessment of anxiety-like behaviours

The elevated plus maze (EPM) test was performed to assess the level of anxiety in animals (Chen et al., 2017). The wooden test apparatus consists of two closed arms (50 × 10 × 40 cm) and two open arms (50 × 10 cm) connected via a central platform (10 × 10 cm) and raised at a height of 50 cm above the floor. Each animal was placed on the central platform facing towards an open arm. Activity was video recorded (Swann SRDVR-16440H, UK) for 5 min. The video files were blinded and coded offline at the end of all experiments using Observer XT 12 (Noldus, the Netherlands). Time spent on open arms was inversely related to the level of anxiety.

## 2.2.8 | Assessment of depression-like behaviour

The sucrose preference test was carried out to assess the depression-like behaviour (Serova, Mulhall, & Sabban, 2017). The animals were separately housed during this test. Here, 24 hr before the test, animals were trained to drink from two bottles each containing 2% sucrose. On the first day of test, the animals were provided with a pre-weighed bottle of 2% sucrose and another containing a pre-weighed volume of tap water. The positions of the bottles were swapped after 24 hr to avoid any side preferences. After 48 hr, both bottles were weighed and sucrose preference was calculated by using the following formula.

$$\text{Sucrose preference (\%)} = \frac{\text{Sucrose consumption}}{\text{Sucrose consumption} + \text{Water consumption}} \times 100.$$

## 2.2.9 | Assessment of cognition

A radial arm maze (RAM) consisting of eight arms (each arm 60 × 10 cm; raised at 50 cm above the floor) was used to assess the reference memory and working memory (WM) of the animals. On four



consecutive days, animals were given two 10-min sessions of habituation to the test apparatus and rules of the test, separated by a 90-min interval. During the first 2 days of habituation, food rewards (1/4 Cheerios<sup>®</sup>, Nestle) were randomly scattered on the floor of the apparatus covering all arms and food troughs at the end of each arm. On the third and fourth habituation day, food rewards were placed only in food troughs of four randomly selected arms (fixed for each animal during the habituation and test day). Food was withdrawn 4–6 hr before the trial (both during habituation and test days) to motivate the animals to locate the rewards and thus perform the task. On the test day, two trials of 10 min were conducted at 90-min interval and the activity of the animals were video recorded for offline blinded coding after the end of experiment. Entry to a non-baited arm was considered as a reference memory error (RME), whereas re-entry to a previously baited arm from which the food was already taken is considered as a working memory error (WME). The mean working memory error or reference memory error was calculated from the two test trials.

## 2.3 | Statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

In Study I, the welfare parameters were analysed using SPSS 24 (IBM SPSS Statistics<sup>®</sup>, UK; RRID:SCR\_002865), while survival data were analysed using GraphPad Prism 6 software (GraphPad Software, Inc., USA; RRID:SCR\_002798). Data obtained from welfare parameters were compared using a three-way ANOVA to observe the main effects of treatment, genotype and time, and their two-way and three-way interactions. If significant two-way interactions were found, Bonferroni post hoc tests were conducted on any Treatment  $\times$  Genotype interactions to assess the effect of CBD treatment on different genotypes (WT/*Scn1a*<sup>-/-</sup>). Bonferroni post hoc tests were also conducted for any significant three-way Treatment  $\times$  Genotype  $\times$  Time interactions to compare the effect of CBD treatment with vehicle treatment at every time point of welfare assessment in both the WT and *Scn1a*<sup>-/-</sup> groups. In all cases, post hoc analyses were corrected for multiple comparisons. Data from 2.2% welfare scores were outliers and were excluded from further analysis ( $\pm 2.5 \times \text{SD}$ ; Miller, 1991). For the survival data, survival curves from *Scn1a*<sup>-/-</sup> vehicle-treated and CBD-treated groups were compared using a Mantel–Cox test. No WT animals died during the study, so survival curves were not compared. All the data are expressed as mean  $\pm$  SEM of  $n$  animals. In all cases,  $P < .05$  is considered as the level of significance.

In Study II, the data were analysed in GraphPad Prism 6 software (RRID:SCR\_002798). Survival curves of *Scn1a*<sup>+/-</sup> vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated group were compared using a Mantel–Cox test. The percentage of animals from the *Scn1a*<sup>+/-</sup> vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups that survived until the end of the study (P52) was compared by Fisher's exact test. Further, data obtained from the co-morbidity assessment were checked for normality by

D'Agostino and Pearson omnibus normality test. Data obtained from rotarod, gait, social interaction (active interaction), elevated plus maze, sucrose preference, and radial arm maze (reference memory error) tests were normally distributed and the differences between the three groups were analysed by one-way ANOVA. If a significant difference was found, then Holm–Sidak post hoc test was conducted among the groups. On the other hand, data obtained from static beam, social interaction (rearing occasions) and radial arm maze (working memory errors) were found to be non-parametric, thus were analysed by Kruskal–Wallis test. Upon observing a significant difference, the Dunn's post hoc test was employed to compare the groups. Multiple comparisons were corrected in all cases. Parametric data are presented in scattered dot plot in the figures and are expressed as mean  $\pm$  SEM. Non-parametric data are presented in box plot in the figures and are expressed as median, min to max, and interquartile range. In all cases,  $n$  = the number of mice in an experimental group, and  $P < .05$  is considered as the level of significance.

## 2.4 | Materials

CBD with batch no. 6046727R (Study I) and 070214 (Study II) were supplied by the GW Pharmaceuticals (Cambridge, UK). Ethanol and Kolliphor<sup>®</sup> were purchased from Sigma-Aldrich (Poole, UK).

## 2.5 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2017), and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

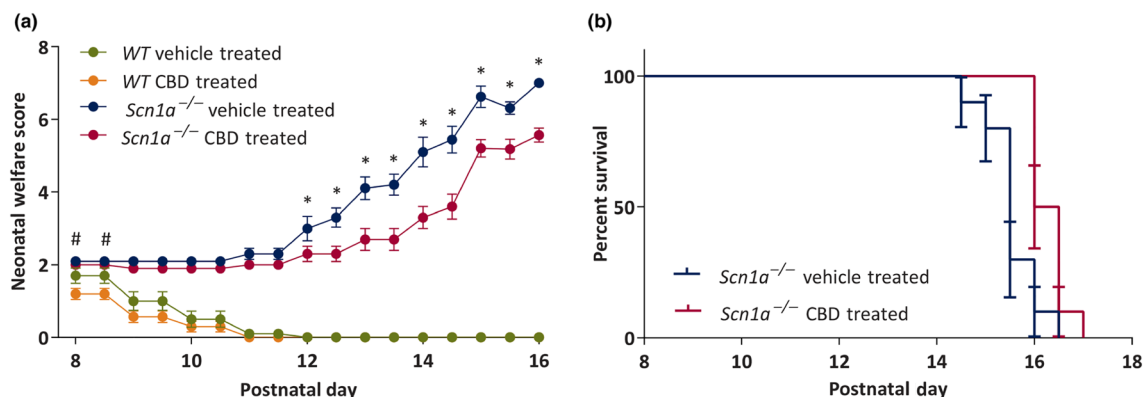
# 3 | RESULTS

## 3.1 | Study I

### 3.1.1 | CBD improved neonatal welfare in *Scn1a*<sup>-/-</sup> mice

In this study, animals ( $n = 10$  per group) were treated with either vehicle or CBD from P8 until P25 or death (whichever was earlier) and welfare was monitored twice daily. The mean TNW scores in WT vehicle-treated, WT CBD-treated, *Scn1a*<sup>-/-</sup> vehicle-treated and *Scn1a*<sup>-/-</sup> CBD-treated groups were respectively  $0.39 \pm 0.04$ ,  $0.24 \pm 0.04$ ,  $3.66 \pm 0.04$  and  $2.85 \pm 0.04$ . Main significant effects of treatment where genotype and time, on TNW scores were found. A significant three-way interaction among Treatment  $\times$  Genotype  $\times$  Time was observed, while significant two-way interactions were observed





**FIGURE 2** Plots showing chronic administration of CBD to wild-type (WT) and *Scn1a*<sup>-/-</sup> mice on neonatal welfare (TNW) score and survival. (A) Neonatal welfare score. CBD significantly improved the TNW score in WT mice from P8 to P8.5 compared to the vehicle-treated WT mice. CBD significantly improved the TNW score in *Scn1a*<sup>-/-</sup> mice from P12 onwards compared to the vehicle-treated *Scn1a*<sup>-/-</sup> mice. Data are expressed as mean  $\pm$  SEM; data were analysed by a three-way ANOVA with Bonferroni's post hoc test; # $P < .05$ ; \* $P < .05$ ; #represents WT vehicle-treated versus WT CBD-treated; \*represents *Scn1a*<sup>-/-</sup> vehicle-treated versus *Scn1a*<sup>-/-</sup> CBD-treated. (b) Survival. CBD treatment significantly ( $P < .05$ ) increased survival compared to the vehicle-treated *Scn1a*<sup>-/-</sup> group. Error bars are SEM; data were analysed by Mantel-Cox test

for Treatment  $\times$  Genotype, Treatment  $\times$  Time and Genotype  $\times$  Time. The post hoc comparison for Treatment  $\times$  Genotype  $\times$  Time interactions revealed that CBD delayed significantly the worsening of welfare scores in *Scn1a*<sup>-/-</sup> mice from P12 to P16 compared to the vehicle-treated *Scn1a*<sup>-/-</sup> mice on respective days (Figure 2a). This post hoc test further showed that CBD significantly improved TNW score in WT animals from P8 to P8.5, that is, in first day of treatment compared to the WT vehicle-treated animals on respective occasions (Figure 2a).

### 3.1.2 | CBD improved survival in *Scn1a*<sup>-/-</sup> mice

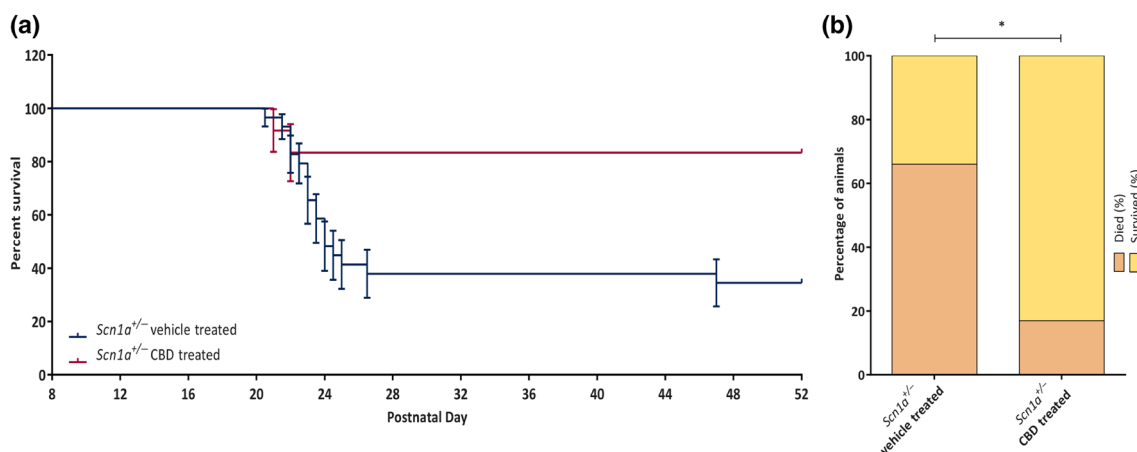
As expected, none of the WT animals died during the study. In the two *Scn1a*<sup>-/-</sup> groups, the median survival in the CBD-treated

*Scn1a*<sup>-/-</sup> mice was significantly higher (16.25 days) compared to the vehicle-treated *Scn1a*<sup>-/-</sup> mice (15.5 days; Figure 2b).

## 3.2 | Study II

### 3.2.1 | CBD prevented premature mortality in *Scn1a*<sup>+/-</sup> mice

The mortality rate was highest between P20 and P27 in *Scn1a*<sup>+/-</sup> mice except for a single animal from the *Scn1a*<sup>+/-</sup> vehicle-treated group which died at P47. We reviewed the recorded video footage and confirmed that tonic-clonic seizures were the cause of death in all cases. Survival was significantly less in *Scn1a*<sup>+/-</sup> vehicle-treated group compared to the *Scn1a*<sup>+/-</sup> CBD-treated group (Figure 3a). Approximately 66% (19 of 29) *Scn1a*<sup>+/-</sup> vehicle-treated animals died before the



**FIGURE 3** Plots showing chronic administration of CBD to *Scn1a*<sup>+/-</sup> mice on survival. (a) Survival of *Scn1a*<sup>+/-</sup> vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated group. CBD treatment significantly increased survival compared to the vehicle-treated *Scn1a*<sup>+/-</sup> group. Error bars are SEM; data were analysed by Mantel-Cox test. (b) Percentage of *Scn1a*<sup>+/-</sup> vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated animals that survived until the completion of experiment (P52). A significantly greater number of CBD treated animals survived until the end of experiment compared to the *Scn1a*<sup>+/-</sup> vehicle-treated animals. Data were analysed by Fisher's exact test. \* $P < .05$

completion of the study in contrast to only 17% (two of 12) *Scn1a*<sup>+/-</sup> CBD-treated animals (Figure 3b). In total, 15 mice died during the dark (active) cycle, and six died during the light (inactive) cycle.

### 3.2.2 | CBD did not exert any adverse effect on motor function

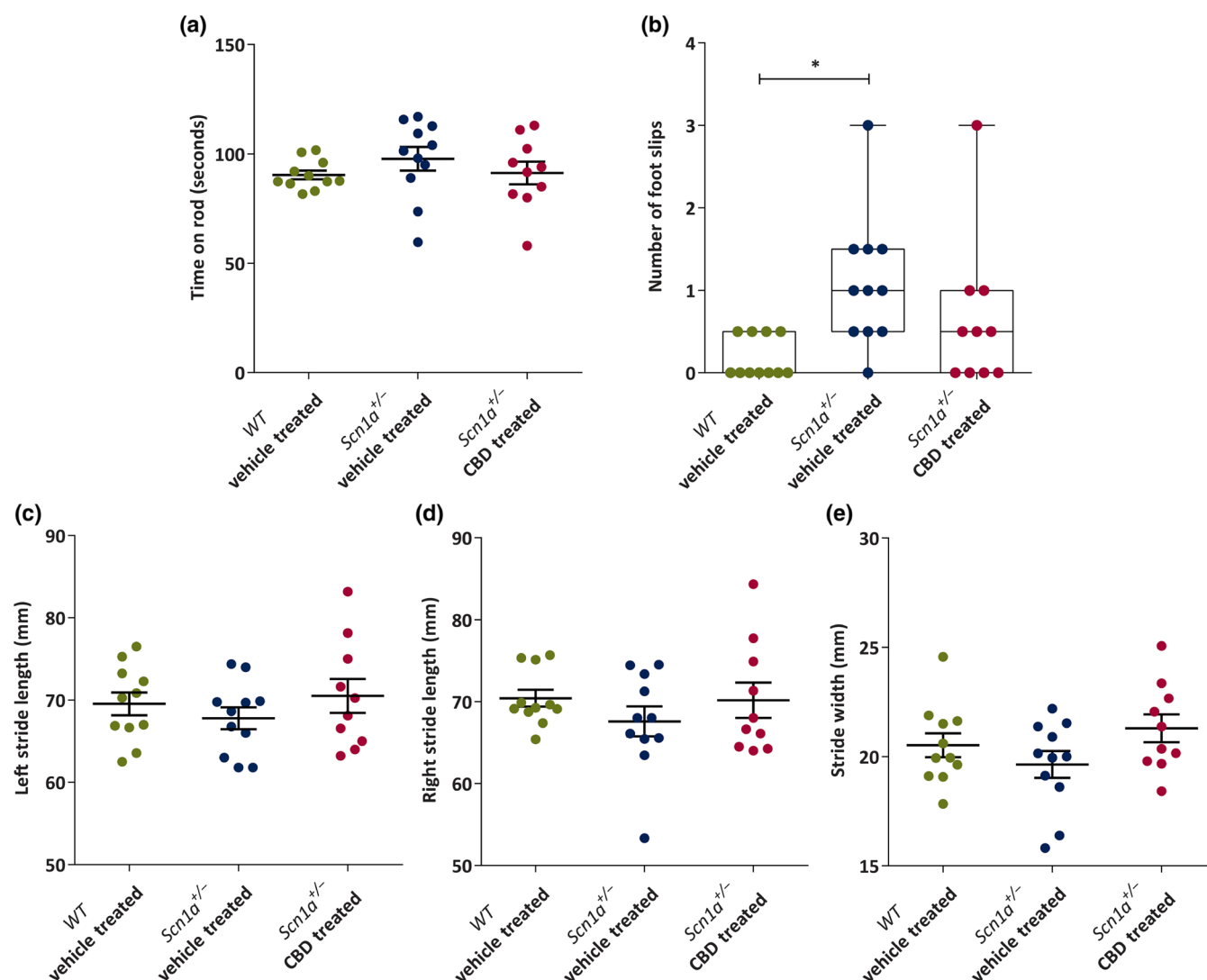
Motor function was assessed by both the accelerating rotarod and static beam test. In the accelerating rotarod test, no significant difference in time spent on rod was observed between the groups (Figure 4a).

On the other hand, in the static beam test, a significant difference in number of foot slips was found among the groups.

*Scn1a*<sup>+/-</sup> vehicle-treated group made significantly more foot slips compared to the WT vehicle-treated group, however no significant difference was observed in between *Scn1a*<sup>+/-</sup> vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups (Figure 4b). A further comparison between the WT vehicle-treated and the *Scn1a*<sup>+/-</sup> CBD-treated groups revealed no significant difference in number of foot slips between these two groups ( $P = .48$ ).

### 3.2.3 | CBD did not produce any gait abnormalities

In the gait test, no significant change was observed for left stride length (Figure 4c), right stride length (Figure 4d) and stride width (Figure 4e), between the three groups.



**FIGURE 4** Dot plots or box and whisker plots showing chronic administration of CBD to *Scn1a*<sup>+/-</sup> mice on motor function and gait. (a) Mean time (seconds) spent on accelerated rotarod by the wild-type (WT) vehicle-treated ( $n = 11$ ), *Scn1a*<sup>+/-</sup> vehicle-treated ( $n = 11$ ) and *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) groups. No difference was observed among the groups. (b) Median number of foot slips made in static beam test. CBD treatment had no adverse effect on foot slips. (c) There were no significant differences in mean left stride length (millimetres) (d) mean right stride length and (e) mean stride width among the groups. All the data are expressed as mean  $\pm$  SEM except foot slips which are expressed as median, min to max and interquartile range (IQR). All data except foot slips were analysed by one-way ANOVA with Holm–Sidak's post hoc test; foot slips data obtained from static beam test were analysed by Kruskal–Wallis test followed by Dunn's multiple comparison test;  $*P < .05$ ; NS, not significant

### 3.2.4 | CBD improved social behaviour of *Scn1a*<sup>+/-</sup> mice

The social interaction test was conducted to assess the active social interaction and rearing behaviour exhibited in the home cage of the test animals.

The time spent on active interaction was significantly differed among the groups. The *Scn1a*<sup>+/-</sup> vehicle-treated animals ( $n = 11$ ) spent significantly less time in performing active interaction with the stranger mouse compared to both WT vehicle-treated ( $n = 11$ ) and *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) animals. The active interaction by the *Scn1a*<sup>+/-</sup> CBD-treated group was similar to the WT vehicle-treated group ( $P = .86$ ).

On the other hand, a significant difference in number of rearing events was observed among the groups, with a significantly higher number of rearing occasions for *Scn1a*<sup>+/-</sup> vehicle-treated animals compared to both WT vehicle-treated or *Scn1a*<sup>+/-</sup> CBD-treated animals (Figure 5b). No difference in rearing events was observed between the WT vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups ( $P = .55$ ).

### 3.2.5 | CBD reduced anxiety-like behaviour in *Scn1a*<sup>+/-</sup> mice

The anxiety of the animals was assessed by the amount of the time spent on the open arms of an elevated plus maze. The time spent on the open arms differs significantly among the groups. The *Scn1a*<sup>+/-</sup> vehicle-treated animals ( $n = 11$ ; Figure 5c) spent significantly less time on the open arms compared to both WT vehicle-treated ( $n = 11$ ) and *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) animals. The time spent on the open arms was not different between WT vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups ( $P = .73$ ).

### 3.2.6 | CBD reduced depression-like behaviour in *Scn1a*<sup>+/-</sup> mice

Depression-like behaviour is inversely correlated with sucrose preference (Murray, Boss-Williams, & Weiss, 2013). In the present study, sucrose preference differed significantly among the groups. *Scn1a*<sup>+/-</sup> vehicle-treated animals ( $n = 11$ ; Figure 5d) had a reduced preference for sucrose in comparison to both WT vehicle-treated ( $n = 11$ ) or *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) animals. Sucrose preference was similar in between the WT vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups ( $P = .36$ ).

### 3.2.7 | CBD improved cognition in *Scn1a*<sup>+/-</sup> mice

The reference memory and WM function in the animals were assessed using an eight-arm radial arm maze test. A significant difference in the number of reference memory error was observed among

the groups. The *Scn1a*<sup>+/-</sup> vehicle-treated group ( $n = 10$ ) made significantly more reference memory error compared to both WT vehicle-treated ( $n = 11$ ) and *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) groups (Figure 5e). No difference in reference memory error was observed between the WT vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups ( $P = .65$ ).

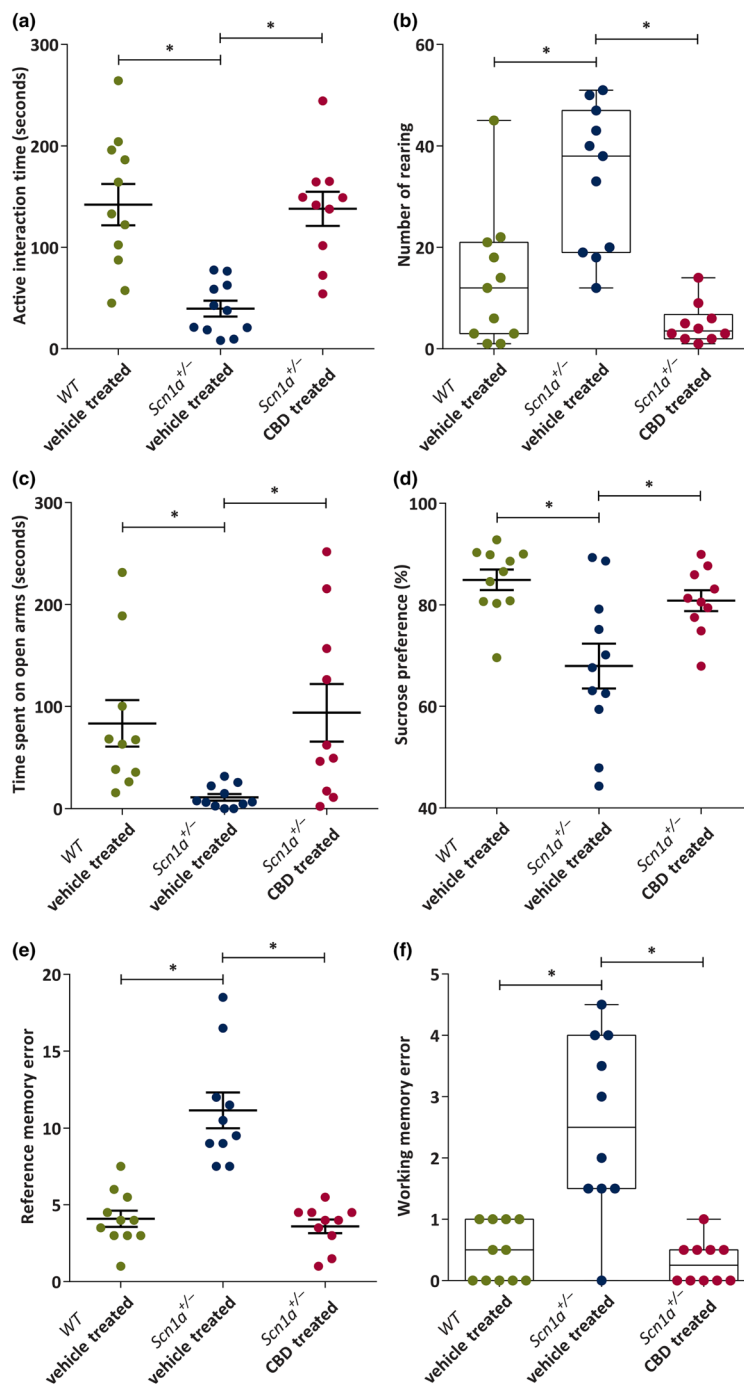
Further, working memory errors were significantly different among the groups. The *Scn1a*<sup>+/-</sup> vehicle-treated group made significantly more working memory errors compared to both WT vehicle-treated and *Scn1a*<sup>+/-</sup> CBD-treated groups (Figure 5f). The working memory errors was not differed in *Scn1a*<sup>+/-</sup> CBD-treated group compared to the WT vehicle-treated group.

## 4 | DISCUSSION

We demonstrate for the first time that chronic administration of CBD extends the survival and improves neonatal welfare of *Scn1a*<sup>-/-</sup> mice. To our knowledge, CBD is the only drug to date which exerts such an effect in this severe model of Dravet syndrome. Given the extreme severity and early mortality observed in this model, it is not possible to study intricate behavioural co-morbidities in the *Scn1a*<sup>-/-</sup> mice. We therefore tested CBD using the *Scn1a*<sup>+/-</sup> mouse model of Dravet syndrome to assess its effect on survival and behavioural co-morbidities. Here, CBD prevented premature mortality and had a disease-modifying action, improving social deficits, reducing anxiety-like and depression-like behaviours and improving memory in the *Scn1a*<sup>+/-</sup> mice.

In Study I, using the 129S *Scn1a*<sup>-/-</sup> knockout model, CBD did not produce any adverse effect on neonatal welfare in WT mice suggesting CBD is safe and well tolerated in healthy neonates. Indeed, CBD actually improved neonatal welfare scores in these mice indicating that CBD provided significant protection against the severe motor deficit and seizure associated deterioration of overall health in this model. In the *Scn1a*<sup>-/-</sup> mice, CBD also improved other welfare parameters such as OT (indicator of pain) and BC score (overall health). Vehicle-treated *Scn1a*<sup>-/-</sup> mice died prematurely as expected with this severe epilepsy phenotype (Kalume et al., 2007; Yu et al., 2006) and CBD treatment significantly extended their survival. This result is remarkable as other AEDs, such as [diazepam](#), have failed to extend survival in this model with only hand-feeding previously observed to extend survival (Yu et al., 2006). No differences in weight (see Data S1) between CBD-treated *Scn1a*<sup>-/-</sup> mice and vehicle-treated *Scn1a*<sup>-/-</sup> mice were seen and CBD induced extension of survival is therefore unlikely to be due to increased feeding. We speculate that the extension of survival is due to the anticonvulsant and disease-modifying properties of CBD, which also lacks the sedating effect of other anticonvulsants used in this model. Indeed, a previous study has established the anticonvulsant effect of CBD in a similar, but less severe, model with haploinsufficiency of Nav1.1 channels (Kaplan et al., 2017), a model which we adopted in Study II.

Given the positive results with CBD in the *Scn1a*<sup>-/-</sup> model of Dravet syndrome, we then conducted a comprehensive investigation into survival and co-morbidities associated with Dravet syndrome



**FIGURE 5** Dot plot or box and whisker plots showing the effect of chronic administration of CBD to *Scn1a*<sup>+/-</sup> mice on active social interaction, rearing, anxiety-like and depression-like behaviours, and cognition. (a) Mean time (seconds) spent on active interaction in social interaction (SI) test by the wild-type (WT) vehicle-treated ( $n = 11$ ), *Scn1a*<sup>+/-</sup> vehicle-treated ( $n = 11$ ), and *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) groups. CBD significantly increased the active interaction time compared to the *Scn1a*<sup>+/-</sup> vehicle-treated group. (b) Median number of rearing made in social interaction test. CBD treatment significantly reduced the number of rearing compared to the *Scn1a*<sup>+/-</sup> vehicle-treated group. (c) Mean time (second) spent on open arms in elevated plus maze (EPM) test. CBD treatment significantly increased the time spent on the open arms of EPM compared to the *Scn1a*<sup>+/-</sup> vehicle-treated group. (d) Mean sucrose preference (%) in sucrose preference test. CBD significantly increased the sucrose preference compared to the *Scn1a*<sup>+/-</sup> vehicle-treated group. (e) Mean reference memory errors (RMEs) in WT vehicle-treated ( $n = 11$ ), *Scn1a*<sup>+/-</sup> vehicle-treated ( $n = 10$ ) and *Scn1a*<sup>+/-</sup> CBD-treated ( $n = 10$ ) groups in radial arm maze (RAM) test. CBD significantly improved RME compared to the *Scn1a*<sup>+/-</sup> vehicle-treated group. (f) Median working memory errors (WMEs). CBD treatment significantly improved working memory errors compared to the *Scn1a*<sup>+/-</sup> vehicle-treated group. All the data are expressed as mean  $\pm$  SEM except rearing and working memory errors which are expressed as median, min to max and interquartile range (IQR); all data except rearing and working memory errors were analysed by one-way ANOVA with Holm-Sidak's post hoc test; rearing and working memory errors data obtained from social interaction test and radial arm test respectively were analysed by Kruskal-Wallis post hoc test followed by Dunn's multiple comparison test; \* $P < .05$

using the haploinsufficiency model of Dravet syndrome, the hybrid *Scn1a*<sup>+/-</sup> heterozygote model. In this model, chronic CBD treatment significantly increased the survival of *Scn1a*<sup>+/-</sup> mice in comparison to their vehicle-treated counterparts; as tonic-clonic seizures (persisting for less than a minute), were the reason for death in all *Scn1a*<sup>+/-</sup> mice, we believe the lower mortality in CBD-treated group is evidence that CBD provides protection against seizure related premature mortality. This result is therefore consistent with a previous study using this model where the anticonvulsant effect of CBD was demonstrated (Kaplan et al., 2017). Sudden unexpected death in epilepsy (SUDEP) in humans often occurs during sleep and is a common form of death in childhood epilepsies such as Dravet (Purnell, Thijs, & Buchanan,

2018). However, our data indicate that the vast majority of animals died during the active phase (dark cycle) indicating that death in this model may not be a predictor of sudden unexpected death in epilepsy in humans.

Seizures and premature mortality are not the only concern in Dravet syndrome, co-morbidities such as motor deficits, including abnormal gait are frequently reported (Aljaafari, Fasano, Nascimento, Lang, & Andrade, 2017; Rilstone, Coelho, Minassian, & Andrade, 2012). Several AEDs used in Dravet syndrome such as [phenobarbital](#), valproic acid, [phenytoin](#) and [lacosamide](#) can also have adverse motor effects in epileptic patients (Bainbridge et al., 2017; Ristić et al., 2006; Zaccara, Perucca, Loiacono, Giovannelli, & Verrotti, 2013). In this

study, CBD had no effect on motor function in either WT or *Scn1a*<sup>+/-</sup> mice indicating that, unlike other AEDs used in treatment of Dravet, CBD does not produce any motor deficits as measured by the accelerating rotorod, static beam or gait analysis.

In addition to motor dysfunction, Dravet syndrome is linked to several neuropsychiatric co-morbidities. For example, social deficits, a feature common in autism, are often observed in Dravet syndrome (Berkvens et al., 2015; Li et al., 2011; Wolff, Casse-Perrot, & Dravet, 2006). We investigated the effect of CBD on the social behaviour of the *Scn1a*<sup>+/-</sup> mice. Vehicle-treated *Scn1a*<sup>+/-</sup> mice had significant social deficits compared to their WT counterparts and CBD-treated *Scn1a*<sup>+/-</sup> mice spent significantly more time in active social interaction compared to their vehicle-treated counterparts. These results are in line with the previous studies conducted in this model where social interactions were impaired but restored by stimulation of **GABA<sub>A</sub>** receptors (Han et al., 2012) and by a single dose of CBD (Kaplan et al., 2017). While this is not the first study to demonstrate that CBD can restore social interaction in a model of Dravet syndrome, we are the first to demonstrate this at doses shown to have anticonvulsant effects. We also demonstrate a chronic CBD dosing regimen, similar to that used in Dravet syndrome therapy (Devinsky et al., 2017, 2018), improves social interactions. Interestingly, CBD has been demonstrated to reduce **Δ<sup>9</sup>-tetrahydrocannabinol**-induced (Malone, Jongejan, & Taylor, 2009) and **MK-801**-induced (Gururajan, Taylor, & Malone, 2012) social deficits in rats, indicating this may be a generalised and reproducible effect of CBD.

In addition to impaired social behaviour, anxiety and depression are major problems for patients with Dravet syndrome (Chen et al., 2018; Jain, Subendran, Smith, & Widjaja, 2018; Wang et al., 2018). Although AEDs such as valproate, **lamotrigine**, **clobazam**, **gabapentin** and **oxcarbazepine** have been reported to improve these behavioural co-morbidities, a number of others have been shown to progressively deteriorate them (Chen, Choi, et al., 2017). Anxiety/depression has been previously reported the Dravet syndrome *Scn1a*<sup>+/-</sup> mouse model (Han et al., 2012) and we observed anxiety-like behaviour in the elevated plus maze in vehicle-treated *Scn1a*<sup>+/-</sup> mice, with animals spending significantly less time on the open arm compared to the WT animals; CBD normalised this behaviour. Interestingly, *Scn1a*<sup>+/-</sup> mice also displayed more rearing behaviours compared to the WT animals in the social interaction tests which could be interpreted as a higher level of anxiety compelling them to them try and escape the test situation (Mines, Yuskaitis, King, Beurel, & Jope, 2010). Again, CBD treatment reduced this anxiety-like behaviour in *Scn1a*<sup>+/-</sup> mice. Together, this illustrates that chronic CBD treatment has anxiolytic potential in this model of Dravet syndrome. This is the first study to suggest an anxiolytic effect of CBD in an epilepsy model. This is consistent with reports of anxiolytic effects of CBD in a chronic unpredicted stress model in mice using both elevated plus maze and novelty suppressed feeding test (Campos et al., 2013; Campos & Guimarães, 2009; Fogaca, Campos, Coelho, Duman, & Guimaraes, 2018) where an action of CBD to inhibit **TRPV1**, **CB<sub>1</sub>** and **CB<sub>2</sub>** was implicated respectively. Anxiolytic effects of CBD have also been observed in a mouse

model of neuropathic pain where inhibition of **5-HT<sub>1A</sub>** receptors was implicated (De Gregorio et al., 2019).

To investigate depression-like behaviours, we employed the conventional sucrose preference test in the *Scn1a*<sup>+/-</sup> mice (Serova et al., 2017). This task is based on the assumption that depression-like behaviour in rodents inversely correlates with sucrose preference (Murray et al., 2013). Here, vehicle-treated *Scn1a*<sup>+/-</sup> mice showed typical depression-like behaviour, that is, a reduced preference to sucrose over water when compared to the WT mice with chronic CBD-treatment normalising sucrose preference. An antidepressant-like action of CBD has previously been documented in genetic (Shoval et al., 2016), olfactory bulbectomy (Linge et al., 2016) and chronic unpredictable stress (Campos et al., 2013) models of depression, but we are the first to demonstrate such effect of CBD in any epilepsy model. Although previous studies in naïve rodents have proposed that CBD exerts an antidepressant action via modulating the **5-HT** neurotransmission or endocannabinoid signalling (Campos et al., 2013; Linge et al., 2016), the underlying pathology of depression in epilepsy is complex and would necessitate a detailed mechanistic study to gain a better understanding on any antidepressant effect of CBD.

Cognitive deficit is also a frequently reported co-morbidity in Dravet syndrome patients (Acha, Perez, Davidson, & Carreiras, 2015; Olivieri et al., 2016; Villeneuve et al., 2014); several domains of cognitive functions including visual attention, executive functions, and verbal, visual and working memories can be impaired in these patients (Acha et al., 2015; Pascualichio et al., 2007; Roebeling et al., 2009). Some AEDs including phenobarbital, phenytoin and topiramate negatively impact cognition in people with epilepsy (Chen, Chi Chow, & Lee, 2001; Mei, Montenegro, Guerreiro, & Guerreiro, 2006; Wandschneider et al., 2017). Here, vehicle-treated *Scn1a*<sup>+/-</sup> mice made significantly more reference memory errors and working memory errors compared to the WT mice in the radial arm maze indicating the presence of spatial memory deficits. We showed that CBD improved both the reference and WM function of *Scn1a*<sup>+/-</sup> mice compared to their vehicle-treated counterparts, to our knowledge, the first report of improving cognitive deficiencies in a model of Dravet syndrome. We have previously reported a similar reversal of cognitive deficits in a rat model of temporal lobe epilepsy (Patra et al., 2019), which may suggest that CBD has a more generalised role in protecting cognitive function in epilepsy, perhaps related to reduction in seizure frequency or severity. Further to this spatial learning memory deficits in a mouse model of Alzheimer's disease were attenuated by CBD in a **CB<sub>2</sub>** receptor-independent manner (Martin-Moreno et al., 2011).

Several hypotheses have been proposed to explain the underlying pathology of memory impairment in epilepsy and seizure is a contributory factor for cognitive decline during the developmental process (Ben-Ari & Holmes, 2006; Khan, Zhao, Miller, & Holmes, 2010). This is consistent with the Dravet model used here as seizures exhibited from an early age. Seizure-related disruption of neural plasticity and cognition has also been demonstrated in other animal models of epilepsies (Lenck-Santini & Scott, 2015; Schubert, Siegmund, Pape, & Albrecht, 2005; Zhou, Lippman Bell, Sun, &



Jensen, 2011). However, removing the cause of seizures does not always restore cognitive deficits in people with epilepsy (Helmstaedter, Kurthen, Lux, Reuber, & Elger, 2003). Strikingly, a caregiver-reported quality of life in childhood epilepsy survey stated an improved memory function in the patients with refractory childhood epilepsy following CBD treatment (Rosenberg, Louik, Conway, Devinsky, & Friedman, 2017). This is consistent with the present study and illustrates the possibility that memory impairment associated with epilepsy/Dravet syndrome may be improved with chronic CBD treatment. We believe these findings warrant further study in the *Scn1a*<sup>+/-</sup> model of Dravet Syndrome to try and ascertain the molecular mechanisms underlying this effect.

## 5 | CONCLUSION

We have established for the first time that the pharmaceutical formulation of CBD (Epidiolex<sup>®</sup>; Epidyolex<sup>®</sup>; therefore, results cannot be directly extrapolated to other CBD formulations) improves neonatal welfare and extends survival in *Scn1a*<sup>+/-</sup> mice. This study is also the first to demonstrate that chronic administration of CBD prevents premature mortality and improves several behavioural co-morbidities, including impaired cognition and social interaction, associated with the *Scn1a*<sup>+/-</sup> mouse Dravet syndrome model. These effects are without the detrimental effects on motor function that are often seen with current pharmacotherapy for this disorder. In light of the recent FDA approval of CBD for treatment of Dravet syndrome in the United States and by the EMA in the EU, we believe that the results obtained from the present indicate that CBD may produce improvements in Dravet syndrome therapy related to survival and co-morbidities in addition to seizure control.

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## CONFLICT OF INTEREST

B.J.W. and M.B. are employees of GW Pharmaceuticals.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for [Design & Analysis](#), and [Animal Experimentation](#), and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

## AUTHORS CONTRIBUTION

P.H.P. designed the study, conducted experiments, analysed and interpreted the data, and wrote the manuscript; E.S. conducted experiments; M.B. and B.J.W. designed the experiments; C.M.W. and

A.J.M. supervised the project, designed the experiments, analysed and interpreted the data and wrote and revised the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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